

# Evaluation of Aro-Tal-AST Complex Protein as a Marker for Differential Diagnosis of *Mycobacterium Avium* Infection

Kapil Gupta, Indu Verma<sup>2</sup>, Gopal K Khuller<sup>2</sup>, Rajiv Mahajan<sup>1</sup>

Departments of Biochemistry and <sup>1</sup>Pharmacology, Adesh Institute of Medical Sciences and Research, Bathinda, <sup>2</sup>Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh, India

## ABSTRACT

**Purpose:** Conventional diagnostic techniques for detecting *Mycobacterium avium* infection are far from satisfactory. As serodiagnostic tests for *M. avium* infection have been shown to be simple and rapid, the present study was carried out to identify and evaluate *M. avium* secretory protein(s) of diagnostic potential. **Materials and Methods:** Initially, by differential immunoblotting, a specific protein band of 45–50 kDa was recognized. Anion exchange column chromatography was used for purification of proteins. After fractionation, blast search was carried out. Further immunoreactivity studies were done with *M. avium* and *Mycobacterium tuberculosis* infected mice sera. Clinical utilization was confirmed by conducting indirect enzyme-linked immunosorbent assay (ELISA) with serum samples from mycobacterial infected patients. **Results:** A complex of three proteins (Aro-Tal-AST) of molecular weight ~48 kDa, shown to be Aro A homologue (Aro), transaldolase (Tal) and aspartate transaminase (AST) by blast search was separated. Immunoreactivity studies of purified complex protein with mice sera confirmed it to be specific for *M. avium* infection. Indirect ELISA with patient samples further confirmed it to be *M. avium* infection specific. **Conclusion:** Aro-Tal-AST protein is specifically recognized by patients infected with *M. avium* and can be used as a marker for simple and rapid ELISA based tests for differential diagnosis of *M. avium* infection in patients with *M. avium* complex (MAC).

**Key words:** Differential diagnosis, Enzyme-linked immunosorbent assay, *Mycobacterium avium*, Secretory proteins

## INTRODUCTION

*Mycobacterium avium* complex (MAC) consists predominantly of two species: *M. avium* subspecies *avium* and *M. avium* subspecies *intracellulare*.<sup>[1]</sup> With growing incidence of human immunodeficiency virus (HIV) infection, cases of MAC infection are also on rise. Hospital-based estimates suggest that 30–50% of patients with AIDS develop MAC infections.<sup>[2]</sup> Disseminated MAC infection is a severe complication of advanced HIV disease.<sup>[3]</sup> Disease due to the MAC is one of the most important opportunistic pulmonary infections most prevalent in immunocompromised patients.<sup>[4]</sup> In more than 95% patients, AIDS related disseminated MAC infection is caused by *M. avium* subspecies *avium*.<sup>[5]</sup> However, recently, incidence of MAC infection has been found to increase

even in immunocompetent pulmonary disease patients.<sup>[6]</sup> Studies have reported that pulmonary diseases caused by MAC in non-HIV infected persons are as common as pulmonary tuberculosis (TB) in many areas.<sup>[2]</sup>

Early differential diagnosis of *M. avium* is becoming increasingly important because of growing frequency of MAC infection in immunocompetent patients,<sup>[6]</sup> impeding availability of new drugs,<sup>[7]</sup> clinical features resembling TB and difficult chemotherapy as compared to *Mycobacterium tuberculosis*<sup>[4]</sup> and poor prognosis of disseminated MAC disease.<sup>[8]</sup>

The diagnostic criteria currently followed by Revised National Tuberculosis Control Program of India, in accordance with American Thoracic Society guidelines for *M. avium* are based on the radiological findings, sputum smear examination along with clinical manifestations. All the features are variable, non-specific and can also be produced by co-existing lung diseases or by other opportunistic infections in HIV seropositive individuals or in early stages of TB by *M. tuberculosis*. Moreover,

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#### Address for correspondence:

Dr. Indu Verma, E-mail: [drinduverma@rocketmail.com](mailto:drinduverma@rocketmail.com)

demonstration of acid-fast bacilli in sputum and bronchoalveolar lavage is same in both *M. tuberculosis* and *M. avium* infections,<sup>[9]</sup> but due to different chemotherapy for the two diseases, prompt distinction is essential and warrants serious consideration for the development of rapid and specific methods for the differential diagnosis of *M. avium* disease from *M. tuberculosis*.

Till date, several diagnostic methods and techniques for differential diagnosis of *M. avium* infection like biochemical tests based diagnostic procedures, <sup>125</sup>I-labeled cDNA probe assay, phenotypic identification of culture by hybridization protection assay, nucleic acid based approaches and ESAT-6 polymerase chain reaction (PCR) primers have been reported.<sup>[10-14]</sup> These methods have their own limitations; they are complex, not very specific, require the growth of mycobacterial cultures from patient's specimen which is time consuming or require radioisotopic facilities, restriction enzymes and extensive instrumentation. So, the development of simple and rapid diagnostic method for differential diagnosis of *M. avium* infection is the need of the hour.

Serodiagnostic techniques, based upon the recognition of species-specific proteins secreted by actively growing mycobacterial bacilli in the culture filtrate (CF), have been advocated to be simple, easy, cost-effective and rapid methods.<sup>[15]</sup> In addition, this is a highly sensitive technique capable of detecting mycobacterial antigens at a concentration of 10<sup>-9</sup>/ml.<sup>[16]</sup> Till date, 14 kDa protein and 81.6 kDa (KatG) protein have been recognized to be helpful in the differential diagnosis of *M. avium* infection.<sup>[17,18]</sup> Taking a clue from these findings, the present study was designed to identify, isolate and purify *M. avium* specific secretory proteins and to evaluate the role of these proteins in the diagnosis of *M. avium* infection, particularly in patients with MAC infection.

## MATERIALS AND METHODS

The present study was an ethically designed study, approved by Institutional Animal Ethical Committee and Institutional Human Ethical Committee. All animal experiments were conducted in accordance with the guidelines laid down by Institutional Animal Ethical Committee. Care of animals was according to the ethical committee guidelines and guidelines issued by CPCSEA.

### Growth of *M. avium* subspecies *avium*, *M. tuberculosis* H<sub>37</sub>Rv, *Mycobacterium bovis* BCG and isolation of culture filtrate proteins

Culture filtrate proteins (CFPs) of *M. avium* subspecies

*avium* (MTCC 1723 IMTECH, NCTC 8551 London), *M. tuberculosis* H<sub>37</sub>Rv (NCTC London) and *Mycobacterium bovis* Bacille Calmette Guérin (BCG) were isolated by growing the bacilli in modified Youman's liquid synthetic medium as a stationary pellicle culture.<sup>[19]</sup> Bacilli were harvested after 4–5 weeks, supernatants were filter sterilized (0.22 µm pore size membrane filter), desalted and concentrated 100 times by ultrafiltration on an amicon YM-3 membrane (Millipore, Bedford, MA, USA). These mycobacterial CFPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–12% resolving gel followed by silver staining.<sup>[20-21]</sup>

### Infection of animals and isolation of antisera

Forty BALB/c mice of either sex (20–25 g body weight, 4–5 weeks) were housed in cages kept in negative pressure regulated animal isolators and were fed on standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. Antisera against various mycobacterial species for comparative enzyme-linked immunosorbent assay (ELISA) and western immunoblot studies were obtained by infection of mice with the above-mentioned bacilli. Briefly, mice (10 in each group) were infected with 1×10<sup>6</sup> bacilli through intravenous route and bled in the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup> and 10<sup>th</sup> weeks post infection. Serum was separated and stored at –20°C till further use.

### Purification of protein

*M. avium* CFPs were separated on the basis of charge using anion exchange column chromatography technique using DEAE sepharose CL-6B matrix. After the washing of column with two-bed volume of equilibration buffer to remove the unbound proteins, the bound proteins were eluted using step gradients of NaCl. Approximately 5–10 mg protein of the desired chromatography gradient containing specific protein of interest was resolved and purified by high-resolution preparative SDS-PAGE (Hoefer SE 600, Amersham Pharmacia, Biotec Inc., San Francisco, CA, USA) (16 cm×4 cm×1.5 mm) at 250 V using Tris-glycine (25 mM, 192 mM) as electroelution buffer. The protein concentration was estimated by micro BCA (Sigma, St. Louis, MO, USA) method and purified protein was analyzed by SDS-PAGE followed by silver staining and stored at –20°C till further use.

### N-terminal sequencing of purified protein

The purified protein was subjected to N-terminal sequencing (ladder sequencing, concentration dependent)

using liquid chromatography-mass spectrometry (LC-MS-MS; Bruker Daltonics Inc., Billerica, MA 01821, USA). The sequence so obtained was carried for blast search to detect the homology with other mycobacterial species.

### Study population

HIV seronegative, pulmonary TB patients ( $n=100$ ) with sputum/bronchoalveolar lavage sample smear positive for acid-fast bacilli (HIV<sup>-ve</sup> TB<sup>+ve</sup> patients)

HIV seropositive patients ( $n=54$ ) with disseminated or extra pulmonary TB having CD4<sup>+</sup> T cells <100 cells /l (HIV<sup>+ve</sup> TB<sup>+ve</sup> patients)

HIV seropositive patients ( $n=20$ ) without any radiological or bacteriological evidence of tuberculosis and with CD4<sup>+</sup> T cell count >100 cells / $\mu$ l (HIV<sup>+ve</sup> TB<sup>-ve</sup> patients)

BCG vaccinated, HIV seronegative healthy volunteers ( $n=20$ )

For detection of antibodies present against *M. avium* specific proteins, 1 ml of blood was withdrawn from all participants, after taking informed written consent.

### Evaluation of diagnostic potential of *M. avium* specific proteins for the identification of *M. avium* disease

To check the significance of *M. avium* specific proteins in the diagnosis of *M. avium* infection in the clinical setup, *M. avium* infected patients were selected from HIV positive population on the basis of culture positivity on the culture media and by a battery of biochemical tests. The *M. avium* specific secretory proteins were subjected to indirect ELISA with 1:100 dilution of sera of HIV<sup>-ve</sup> TB<sup>+ve</sup> patients, HIV<sup>+ve</sup> TB<sup>+ve</sup> patients, HIV<sup>+ve</sup> TB<sup>-ve</sup> patients, *M. avium* antisera, *M. tuberculosis* antisera and healthy individual's sera to check their specificity for the diagnosis of *M. avium* infection.

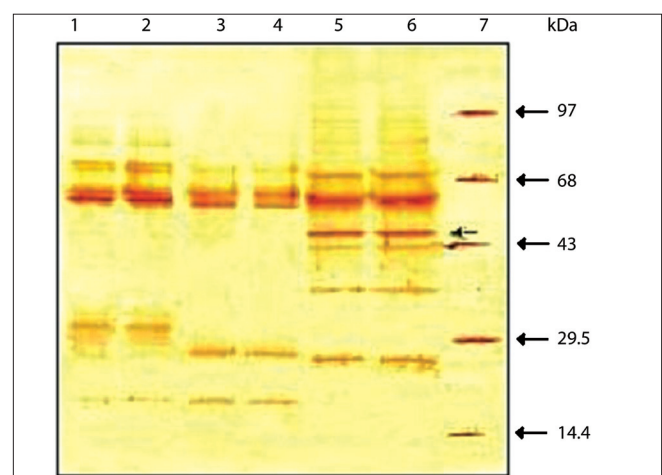
## RESULTS AND DISCUSSION

The 4-week stationary culture of *M. avium* subspecies *avium*, *M. tuberculosis* H<sub>37</sub>R<sub>v</sub> and *M. bovis* BCG isolated the CFPs with a mean yield of  $15 \pm 2$  mg/l of the secretory proteins. The CFPs resolved on 12% denaturing gel showed the presence of protein bands ranging from a molecular weight of 6 to 97 kDa on SDS-PAGE, but in *M. avium* CF, the protein band in the regions of 45–50 kDa seemed to be specifically present and absent from rest of mycobacterial species.

Immunoblotting of *M. avium* antisera with *M. bovis* BCG, *M. tuberculosis* H<sub>37</sub>R<sub>v</sub> and *M. avium* CFPs also indicated the presence of a number of common protein bands in all the mycobacterial species. However, *M. avium* antisera immunoblotted with *M. avium* CF showed a specific protein band of 45–50 kDa that was not evident in others [Figure 1].

Anion exchange column chromatography resulted in the elution of an immunodominant protein of ~48 kDa in 150 mM elution gradient. Comparative immunoblotting of ~48 kDa protein of 150 mM elution gradient with *M. avium* antisera (taken as positive control) and TB patient's sera resulted in the recognition of these proteins only with *M. avium* antisera and not with TB patient's sera or *M. tuberculosis* antisera, thus depicting the specificity of these proteins for *M. avium* infection [Figure 2].

On LC-MS-MS, the ~48 kDa protein was found to be a complex protein demonstrating three peaks in the mass spectra. Molecular mass of these three proteins was 47.3, 50.9 and 55.7 kDa with the number of amino acids being 463, 469 and 515, respectively. The first protein designated as Aro A homologue protein (Aro) showed 92% homology with *M. avium* paratuberculosis and 75.59% homology with *M. tuberculosis* starting at 31<sup>st</sup> amino acid. The second protein designated as Tal protein (putative transaldolase) showed 79.53% homology with *M. avium* paratuberculosis and 66.73% homology with *M. tuberculosis* starting at 97<sup>th</sup> amino acid, while with the third protein, i.e. aspartate transaminase (AST), the homology was 82.91% and 68.93% with *M. avium* paratuberculosis and *M. tuberculosis*, respectively, starting at the 88<sup>th</sup> amino acid [Table 1]. The protein was



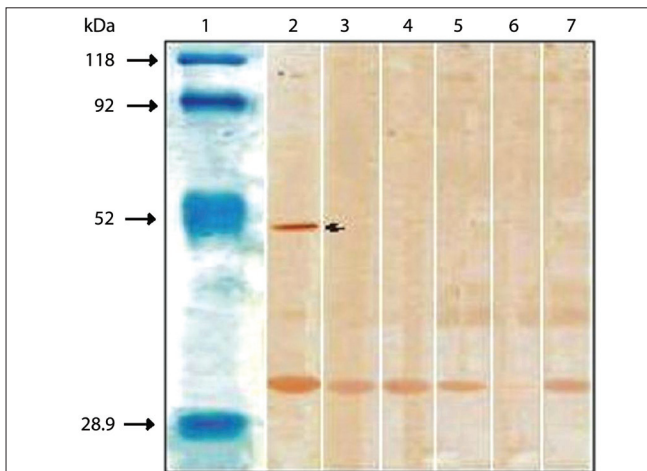
**Figure 1:** Western immunoblotting of *M. avium* antisera with different mycobacterial culture filtrates (CFs). Lanes 1 and 2: With *M. bovis* BCG CF; Lanes 3 and 4: With *M. tuberculosis* CF; Lanes 5 and 6: With *M. avium* CF; Lane 7: Standard molecular weight markers. (The black arrow within immunoblot indicates the presence of specific protein band of 45–50 kDa)



**Table 1: Characterization of purified *M. avium* specific Aro-Tal-AST secretory protein**

Character	Mw in kDa	AAs	PI	Homology with (based on BLAST search) (%)		N-terminal sequence	Protein identity
				<i>M. avium</i> paratuberculosis	<i>M. tuberculosis</i>		
Complex of three proteins	i-47.3	463	5.50	92.00	75.59	VAEPPGAGPW	Aro A homologue (Aro)
	ii-50.9	469	6.32	79.53	66.73	LWRGWCPRA	Transaldolase (Tal)
	iii-55.7	515	8.38	82.91	68.93	MSGDPLRPAP	Aspartate transaminase (AST)

Mw=molecular weight, AAs=amino acids, PI=isoelectric pH



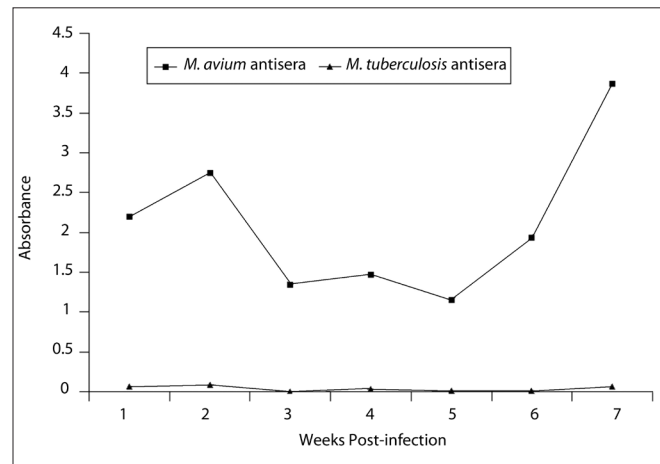
**Figure 2:** Comparative immunoblotting of 150 mM elution gradient of *M. avium* CF with *M. avium* antisera and TB patient's sera. Lane 1: Standard pre-stained molecular weight markers; Lane 2: Immunoblotting with *M. avium* antisera; Lanes 3–7: Immunoblotting with TB patient's sera. (The black arrow within the immunoblot indicates the presence of specific protein band of ~48 kDa)

designated as Aro-Tal-AST complex protein. These data show that the N-terminal sequence of all the three proteins are different from other mycobacterial species.

Immunoreactivity studies of purified Aro-Tal-AST complex protein with *M. avium* and *M. tuberculosis* infected mice sera demonstrated the presence of Aro-Tal-AST antibodies only in *M. avium* antisera [Figure 3] and confirmed the specificity of *M. avium* Aro-Tal-AST complex protein for the diagnosis of *M. avium* infection.

Kinetic expression studies performed by immunoblotting *M. avium* CF with *M. avium* antisera collected in 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup>, 12<sup>th</sup> weeks after experimental *M. avium* infection in mouse model showed that Aro-Tal-AST complex protein was recognized as early as from 2<sup>nd</sup> week post infection [Figure 3].

Significance of purified *M. avium* Aro-Tal-AST complex protein in the diagnosis of MAC in the clinical setup was confirmed by indirect ELISA with the patients with MAC bacteremia, selected from HIV<sup>+</sup> population with disseminated mycobacterial disease on the basis of blood culture by lysis centrifugation method, followed by a battery of biochemical tests. Out of 54 blood samples of



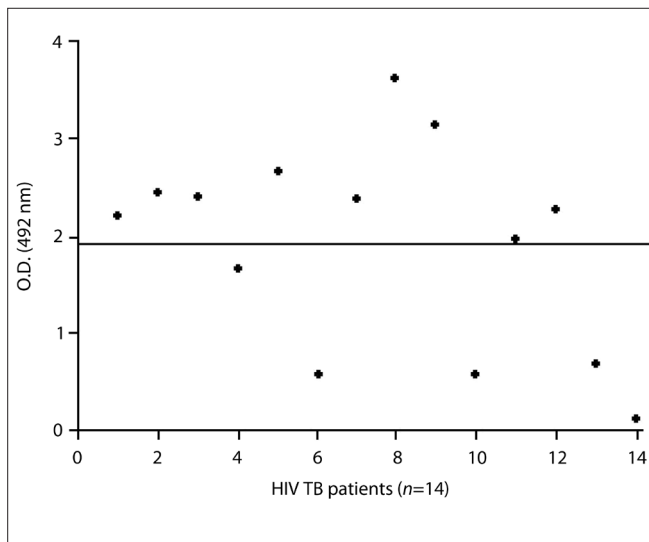
**Figure 3:** Recognition of *M. avium* Aro-Tal-AST complex protein in *M. avium* infection in mouse model

HIV patients with disseminated mycobacterial infection, 14 samples were mycobacterial culture positive, and of these 14 samples, 10 were MAC and 4 were *M. tuberculosis* positive on the basis of biochemical tests. When purified *M. avium* Aro-Tal-AST complex protein was subjected to indirect ELISA with the serum samples (1:100 dilutions), of these 14 patients, 9 out of 10 MAC positive patients had antibodies to recognize the protein, giving a percent recognition of 90%. Four samples that were positive for *M. tuberculosis* did not recognize Aro-Tal-AST complex protein, confirming that this protein is specific to *M. avium* infection [Figure 4].

Further, *M. avium* Aro-Tal-AST complex protein based ELISA in the serum samples from 100 pulmonary TB patients demonstrated that 99/100 patients showed no reactivity, thus confirming the specificity of *M. avium* Aro-Tal-AST complex based serodiagnostic assay [Figure 5]. Also, the absence of anti-Aro-Tal-AST complex antibodies in healthy BCG vaccinated individuals further confirmed the recognition of this protein only during active disseminated MAC disease and showed that it is not affected by prior BCG vaccination/exposure to environmental mycobacteria.

## CONCLUSION

Aro-Tal-AST complex protein based ELISA has an overall

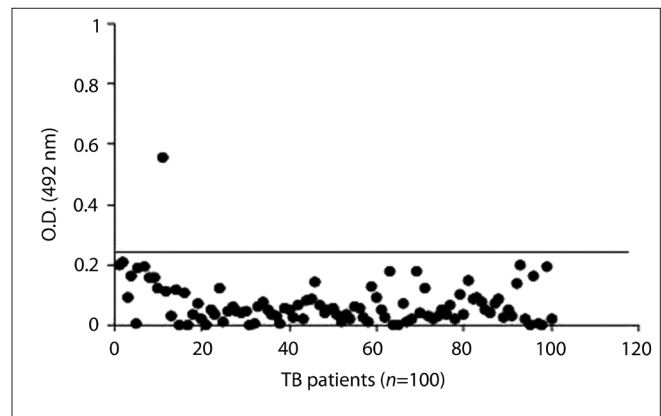


**Figure 4:** Detection of antibodies in serum samples of HIV seropositive mycobacterial culture positive patients against Aro-Tal-AST complex proteins. The solid horizontal line shows the cut-off value, i.e. mean absorbance of HIV positive TB negative, HIV negative TB positive, HIV negative TB negative patients + 2.5 SD

sensitivity of 90% and specificity of 99% in diagnosing *M. avium* infection. Moreover, this test can also be applied for HIV positive/AIDS patients, who do not respond to T-cell based assays due to very low CD4<sup>+</sup> T-cell counts. Further, this protein is recognized as early as 2<sup>nd</sup> week post infection. These data clearly demonstrate that the *M. avium* Aro-Tal-AST complex protein is able to differentially diagnose disseminated MAC disease in HIV infected population at an early stage and the results obtained with antibody-based easy and rapid ELISA test correlate with those of conventional time-consuming blood culture and biochemical tests.

Of the 40 patients with disseminated *Mycobacterium* infection but blood culture negative, 4 had anti-Aro-Tal-AST antibodies in their serum, but as they were not differentially diagnosed to be *M. avium* infected clinically, nothing can be ascertained. As no other test can specifically differentiate *M. avium* from *M. tuberculosis*, it may be possible that these patients were *M. avium* infected, but neither were they diagnosed clinically nor they were blood culture positive. Therefore, presently, we cannot say surely from the available data if Aro-Tal-AST can be considered a marker for MAC infection in patients with negative biochemical tests.

This study clearly indicates that the development of *M. avium* Aro-Tal-AST complex protein based diagnostic ELISA could be of great help to the clinicians in the timely diagnosis and treatment of disseminated MAC disease in HIV patients.



**Figure 5:** Evaluation of seroreactivity of Aro-Tal-AST complex protein with the serum samples of smear positive TB patients. The solid horizontal line shows the cut-off value [mean OD of healthy individuals + 2.5 SD, i.e. 0.0675 + 2.5 (0.068)]

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